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Identification of a Glucose Permease from *Mycobacterium smegmatis* mc^2 155

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Key Words
Sugar transport · Membrane topology · Mycobacterium · Pathogenicity · Bacterial lifestyle · Carbon regulation

Abstract
We report here the molecular identification of a glucose permease from *Mycobacterium smegmatis*, a model organism for our understanding of the life patterns of the major pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae*. A computer-based search of the available genome of *M. smegmatis* mc^2 155 with the sequences of well-characterized glucose transporters revealed the gene *msmeg4187* as a possible candidate. The deduced protein belongs to the major facilitator superfamily of proton symporters and facilitators and exhibits up to 53% of amino acid identity to other members of this family. Heterologous expression of *msmeg4187* in an *Escherichia coli* glucose-negative mutant led to the restoration of growth on glucose. The determination of the biochemical features characterize MSMEG4187 (GlcP) as a high affinity (K_m of 19 μM), glucose-specific permease. The results represent the first molecular characterization of a sugar permease in mycobacteria, and thus supply fundamental data for further in-depth analysis on the nutritional lifestyle of these bacteria.
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Introduction

Mycobacterium tuberculosis remains a serious threat to human health [Kaufmann, 2006]. About one-third of the world's population is thought to be infected with this pathogenic microorganism. In spite of significant investments in research, the mechanisms of its pathogenicity are still not fully understood. One aspect that is inextricably linked to pathogenicity is nutrition. This has been recently demonstrated by the identification of central metabolic genes, including carbohydrate transporter genes, which are essential for entering into and proliferating inside the macrophage [Joshi et al., 2006]. The current literature reveals limited information about the intermediary metabolism, utilization of organic compounds in the process of growth, and carbon catabolite control of mycobacteria. Efforts have been undertaken to understand virulence in mycobacteria by using the fast-growing *Mycobacterium smegmatis*. This model organism is infrequently reported as pathogenic and causes skin or soft tissue infections [Brinton et al., 1991; Lustgarten, 1884].

We reported previously the identification of a glucose kinase gene from *M. smegmatis* mc^2 155, which could be a potential signalling factor for carbon regulation [Pimentel-Schmitt et al., 2006]. Since the transport of a carbon source represents, for many pathways, the decisive

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step for the flux through the pathway [Ruyter et al., 1991], we aimed to unravel the glucose uptake system(s). A putative glucose transporter that is encoded by the gene *msmeg4187* was recently detected by us in the course of a genome analysis of the carbohydrate permeases of *M. smegmatis* and *M. tuberculosis* [Titgemeyer et al., 2007].

In this communication, we demonstrate the function of the possible glucose transporter by heterologous complementation in an appropriate mutant of *Escherichia coli*. Furthermore, we provide biochemical details on the catalytic function of GlcP (MSMEG4187) and present a detailed topology model that includes the coordinates of conserved amino acid residues among related bacterial glucose permeases. $GlcP^{Msm}$ is to the best of our knowledge the first molecular described sugar permease in the genus mycobacteria.

Results

msmeg4187 Encodes a Putative Glucose Permease

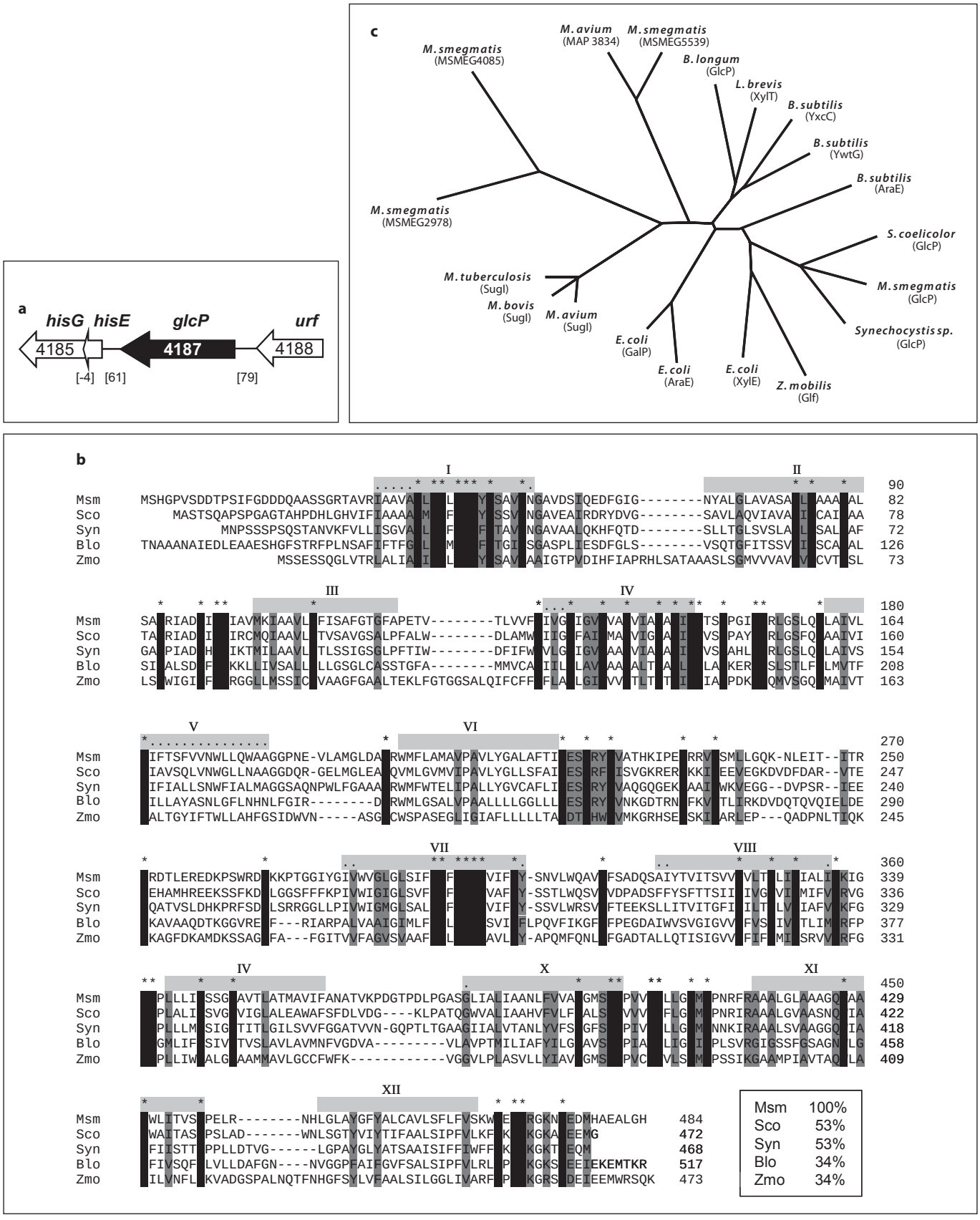
The protein database of the *M. smegmatis* genome was scanned with several known glucose permease protein sequences using BLASTP as implemented at The Institute for Genomic Research (TIGR) [Parche et al., 2006; van Wezel et al., 2005]. We found only one gene (*msmeg4187*) that encodes a predicted glucose permease of the major facilitator super family (MFS) [Pao et al., 1998]. The deduced protein shares 53% amino acid identity to the well-characterized glucose permease GlcP of *Streptomyces coelicolor* [Titgemeyer et al., 2007; van Wezel et al., 2005]. Analysis of the gene locus of *msmeg4187* (hereafter named *glcP*) indicated that the gene is transcribed as a monocistronic mRNA (fig. 1). *glcP* is flanked downstream by a putative dicistronic *hisEG* operon of genes involved in histidine biosynthesis, and upstream by an unknown open reading frame. While the *hisEG* operon is conserved in mycobacteria, it appears that the slow-growing mycobacteria, e.g. *M. tuberculosis*, *M. bovis*, and *M. avium*, do not have an obvious homologue of *glcP*.

The multiple alignment shown in figure 1b includes four homologous glucose permeases which have been experimentally described [Parche et al., 2006; van Wezel et al., 2005; Weisser et al., 1995; Zhang et al., 1989]. The range of protein identity is from 34 to 53%, showing 67 conserved residues in these sequences. The two typical signature sequences of the MFS are found in $GlcP^{Msm}$ at positions 126–151 (VgGIGvGVasviapayiaETsppgiR) and 331 to 348 (AIALIDKIGRKplligS). Hydrophobicity profiles showed that all five sequences have 12 predicted transmembrane helices that are at equivalent positions (fig. 1b). To establish the phylogenetic relationships of $GlcP^{Msm}$, we selected 19 protein sequences of the MFS which have been characterized by experimental investigation and those from diverse mycobacteria (fig. 1C). $GlcP^{Msm}$ clusters with the *S. coelicolor* homolog and the one from the cyanobacterium *Synechocystis* [van Wezel et al., 2005; Zhang et al., 1989]. Interestingly, the xylose permease XylE of *E. coli* is more closely related to $GlcP^{Msm}$ than expected, while the bifidobacterial GlcP is more distant but closely associated with another xylose permease, XylT of *Lactobacillus brevis* [Chaillou et al., 1998]. This suggests that glucose and xylose transporter genes may have evolved following two different branches from the common origins.

Heterologous Expression of GlcP in E. coli

The sequence of *msmeg4187* was amplified from chromosomal DNA and cloned into plasmid pSU2719 in orientation of the *lacZ* gene, yielding plasmid pFT283 (*glcP*$^+$). The plasmid was introduced into *E. coli* LJ140 (Δ*ptsHIcrr::kan*), which does not transport glucose to appreciable

Fig. 1. Genetic organization of the *glcP* gene in *M. smegmatis*, protein alignment, and phylogenetic tree. **a** Genetic map. The depicted region of *glcP* (black) in *M. smegmatis* includes the genes *hisG*, *hisE* (both histidine biosynthetic genes), and a gene encoding a protein of unknown function (*urf*, MSMEG4188). The genome of *M. smegmatis* (mc^2 155) was sequenced by TIGR (http://www.tigr.org/). The arrows indicate length and transcriptional orientation of annotated genes which are depicted by the respective *orf* number. Numbers in square brackets show the lengths of intergenic regions in bp. **b** The amino acid sequences of *glcP* in *M. smegmatis* were aligned with representative homologues. Residues identical in all proteins are shaded black and those that are conserved are shown in grey. Above the alignment, the residues present in all proteins are marked by asterisks. Abbreviations and protein designations are as follows. Msm, *M. smegmatis* (MSMEG4187); Sco, *S. coelicolor* (SCO5578); Syn, *Synechocystis sp. PCC6803* (sll0771); Blo, *B. longum* (BL1631); Zmo, *Z. mobilis* (ZMO0366). **c** Phylogenetic tree of the *glcP* family. An unrooted phylogenetic tree was computed with the CLUSTALW software making use of the implemented neighbor joining method with the function for evolutionary distance correction. Evolutionary distances are proportional to the branch length. 19 protein sequences were selected as indicated in the figure. The references for experimentally verified transporters are as follows: Chaillou et al., 1998; Krispin and Allmansberger, 1998; Martin et al., 1994; Parche et al., 2006; van Wezel et al., 2005; Weisser et al., 1995; Zhang et al., 1989.


a
hisG
hisE
glcP
urf
4185
4187
4188
[-4]
[61]
[79]
c
M. smegmatis (MSMEG4085)
M. avium (MAP 3834)
M. smegmatis (MSMEG5539)
B. longum (GlcP)
L. brevis (XylT)
B. subtilis (YxcC)
B. subtilis (YwtG)
B. subtilis (AraE)
M. smegmatis (MSMEG2978)
S. coelicolor (GlcP)
M. tuberculosis (SugI)
M. smegmatis (GlcP)
M. bovis (SugI)
M. avium (SugI)
Synechocystis sp. (GlcP)
E. coli (GalP)
E. coli (AraE)
E. coli (XylE)
Z. mobilis (Glf)
b
Msm
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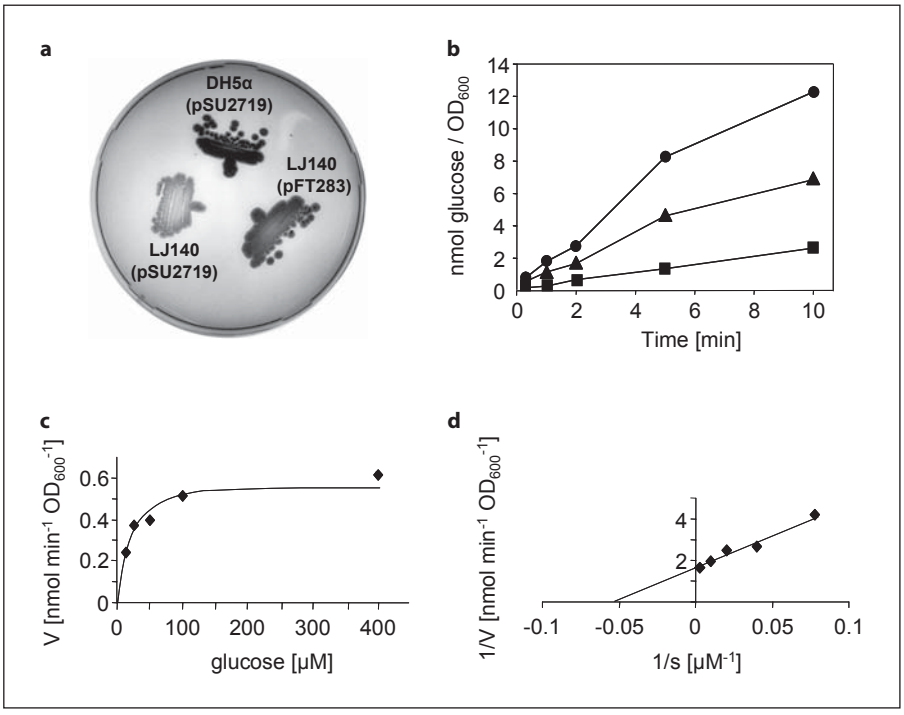

Fig. 2. Heterologous complementation and tranport kinetics. **a** Complementation of the glucose-negative phenotype in *E. coli* LJ140 (pFT283 *glcP*$^+$) is shown on a MacConkey agar base plate supplemented with 50 mM glucose. Pigmented red (here grey) colonies indicate the fermentation of glucose, whereas white colonies reflect a deficiency in glucose fermentation. DH5α (pSU2719) was used as a positive control (dark gray) and LJ140 (pSU2719) as the congenic negative control. **b** Time-dependent uptake of 100 μM [^{14}C]glucose using cells of DH5α(pSU2719) (circles, positive control), LJ140 (pFT283) (triangles) and LJ140 (pSU2719) negative control (squares). Standard deviations of triplicate data points are shown by error bars but are barely visible due to minimal data variation. **c** [^{14}C]glucose uptake at different concentrations were fit to a non-linear regression and (**d**) graphed into a Lineweaver-Burk plot revealing a K_m of 19 μM and V_{max} of 0.63 nmol min^{-1} OD$_{600}^{-1}$.

amounts due to a chromosomal deletion of the *pts* operon. LJ140(pFT283), and several control strains were streaked onto MacConkey agar plates supplemented with 50 mM glucose (fig. 2a). The presence of *M. smegmatis glcP* led to the restoration of glucose fermentation as indicated by the formation of gray colonies. In comparison, the congenic strain LJ140(pSU2719) that carries the same cloning vector without the *glcP* gene grew as white colonies.

GlcP Is a High-Affinity Glucose Permease with Narrow Substrate Specificity

Glucose uptake experiments were performed to further characterize the biochemical features of the GlcP^{Msm} permease (fig. 2b). While LJ140(pSU2719) showed negligible uptake kinetics, transport of glucose was readily detectable when *msmeg4187* was expressed in LJ140(pFT283), reaching about 50% of the intrinsic *E. coli* wild-type glucose transport activity that was monitored in *E. coli* DH5α(pSU2719). A series of uptake experiments with various glucose concentrations was then performed to determine the kinetic parameters of GlcP (fig. 2c, d). We determined an affinity constant (K_m) of 19 μM and a velocity (V_{max}) of 0.63 nmol min^{-1} OD$_{600}^{-1}$. The substrate specificity was investigated by a competitive inhibition of glucose transport. Therefore, we added a hundredfold excess (10 mM) of nonradioactive sugar. While the presence of excess glucose and the derivative 2-deoxyglucose led to a reduction of more than 80% of the initial uptake rate, no or very small effects (<10%) were observed, when methyl α-D-glucopyranoside, xylose, arabinose, fructose, and trehalose were tested. In conclusion, the biochemical analysis of GlcP^{Msm} revealed a high-affinity permease with a narrow substrate specificity.

Discussion

The MFS family is one of the largest transporter families with members in bacteria and in eukaryotes like the GLUT facilitator transporters of mammals [Pao et al., 1998]. The transmembrane proteins of the GLUT family exhibit different substrate specificities, kinetic properties and tissue expression profiles. GLUT1 for example is expressed as a high-affinity permease in erythrocytes, the perineurium of peripheral nerves, and capillary endothelial cells of the blood-brain barrier [Gould et al., 1991; Mueckler et al., 1985; Salas-Burgos et al., 2004]. GLUT2 is a low-affinity glucose transporter, expressed in the liver, pancreas, intestine, and kidneys [Fukumoto et al., 1988; Gould et al., 1991]. GLUT3 and GLUT4 are high-affinity symporters present in brain, heart, muscle, and brown adipose tissue [Fukumoto et al., 1989; Kayano et

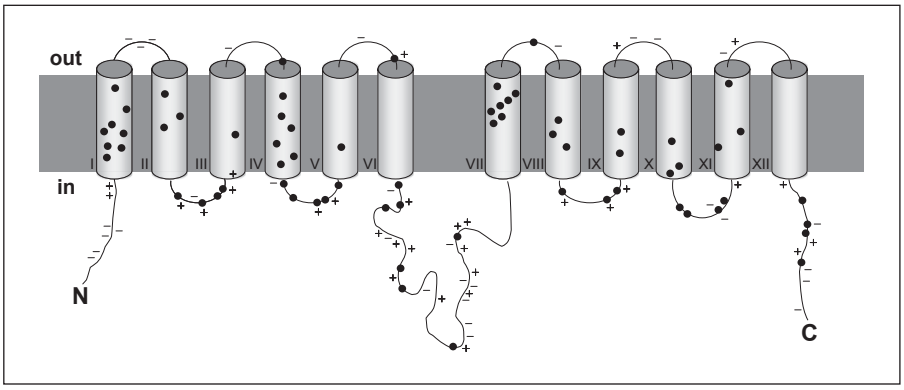

Fig. 3. Topology model of *M. smegmatis* GlcP. The drawing displays the predicted two-dimensional topology structure of GlcP, which is composed of 12 putative transmembrane segments. Conserved residues present in bacterial GlcPs (fig. 1b) are highlighted by black circles. Charged amino acids are marked by (+) and (–), showing a charge distribution of more residues on the inside of a transmembrane helix compared to the outside as defined by the positive-inside rule described by Heijne [1986].

al., 1988; Wood and Trayhurn, 2003]. Thus, this type of glucose permease has obviously been very successful in evolution.

In this study, we report by molecular means the first mycobacterial sugar permease and established that heterologous gene expression of a mycobacterial integral membrane protein in *E. coli* is feasible. The permease is the glucose-specific transport protein GlcP from *M. smegmatis* mc^2 155 encoded by open reading frame *msmeg4187*. $GlcP^{Msm}$ exhibited an apparent K_m of 19 μM when expressed in *E. coli*. This correlates with the corresponding values of 41 μM of *S. coelicolor's* GlcP [van Wezel et al., 2005] and 70 μM of *B. longum's* GlcP [Parche et al., 2006]. $GlcP^{Msm}$ shows the closest phylogenetic relationship to glucose permeases from streptomycetes and cyanobacteria [van Wezel et al., 2005; Zhang et al., 1989], which differ in their substrate specificity for fructose. $GlcP^{Msm}$ was found specific for glucose and 2-deoxyglucose and not for fructose. Thus, it resembles in its substrate specificity profile the homolog $GlcP^{Sco}$ from *S. coelicolor* [van Wezel et al., 2005]. In fact, our recent genome-based compilations of all carbohydrate transport systems of *S. coelicolor* and *M. smegmatis* shows that these two soil-dwelling actinomycetes have many homologous sugar uptake systems in common [Bertram et al., 2004; Titgemeyer et al., 2007].

Our bioinformatic analysis on the membrane topology of $GlcP^{Msm}$ led to the model presented in figure 3. $GlcP^{Msm}$ has a predicted composition of 12 transmembrane helices typical for MFS porters [Pao et al., 1998]. The model was obtained by aligning the predictions of possible transmembrane segments of the five GlcP proteins depicted in figure 1b assuming that they all share the same overall topology. This showed that 12 transmembrane segments are probably present in all of them, while sequence variations in terms of deletions and insertions occurred only in regions which were predicted as flexible loops. Figure 3 displays that helices I, IV, and VII exhibit (with eight, six, and seven conserved residues) the highest conservation suggesting that these may be most relevant for substrate recognition.

Interestingly, no GlcP homolog is present in slow-growing mycobacteria, notably *M. tuberculosis* and *M. leprae* [Titgemeyer et al., 2007]. In these bacteria, glucose may enter either by simple diffusion, which could be efficient enough to meet the nutritional demands of these extremely slow-growing bacteria (generation time of weeks), or through another, yet unknown permease [Titgemeyer et al., 2007]. *M. smegmatis* and also the slow-growing mycobacterial species possess a glucose kinase that converts the incoming glucose to glucose-6-phosphate [Pimentel-Schmitt et al., 2006; Titgemeyer et al., 2007]. Apart from its catalytic function, this enzyme confers global carbon regulation in closely related streptomycetes [Angell et al., 1994]. We could recently show in *S. coelicolor* that glucose kinase binds to glucose permease GlcP [van Wezel et al., 2007]. This protein-protein interaction is thus thought to influence the regulatory function of glucose kinase. For this reason, it would be worthwhile to study, whether GlcP and GlkA of *M. smegmatis* also interact with each other and whether this has similar consequences regarding nutritional control. This in turn would help to understand how nutrition and virulence may be linked in mycobacteria.

Experimental Procedures

Bacterial Strains and Growth Conditions

Cells of *E. coli* were grown at 37°C under vigorous shaking in Luria-Bertani broth (LB) and supplemented with the appropriate antibiotics when required. *E. coli* DH5α served as the host strain for gene cloning purposes [Sambrook et al., 1989]. LJ140 (Δ*ptsHI::*

kan) is a derivative of *E. coli* LJ110 [Zeppenfeld et al., 2000], in which a deletion of the *pts* operon was introduced by common P1(kc) transduction through selection of a gene for kanamycin resistance which substitutes the *pts* locus.

Heterologous Complementation

Chromosomal DNA was prepared from 100-ml cultures grown to saturation [Belisle and Sonnenberg, 1998]. 400 ng was used as template for a polymerase chain reaction with oligonucleotides FS12 ggctgtctagatcattgggccgcaggaagaaga and FPS21 tgcgccgatgggctctagatgccgtcagt (engineered *Hin*dIII and *Xba*I restriction sites are underlined). Primer FS12 contained stop codons in all three reading frames and an artificial ribosome binding site for *glcP* to ensure efficient translation. The amplified DNA fragment was digested and ligated in pSU2719 in the same orientation as the *lacZ* gene [Chandler, 1991]. The ligation mixture was transformed into DH5α. Recombinant plasmids were isolated and confirmed by DNA sequencing. The resulting $glcP^{Msm}$ expression vector was designated pFT283. LJ140 was transformed with pFT283 or pSU2719 (control), and transformant colonies were streaked out on MacConkey agar plates supplemented with 50 mM glucose in order to examine heterologous complementation of the glucose transport-negative phenotype.

Transport Assays

Cells of *E. coli* LJ140 bearing either plasmid pSU2719 or pFT283 $glcP^{+}$ and *E. coli* DH5α(pSU2719) were grown at 37°C in 20 ml LB supplemented with chloramphenicol to an OD_{600} of 1.0. Cells were pre-equilibrated to 37°C before uptake was initiated by the addition of [^{14}C]glucose at a final concentration of 100 μM (100,000 cpm/ml) [Nothaft et al., 2003]. Samples of 0.5 ml were taken at different time points, rapidly filtered through nitrocellulose filters, and washed with 5 ml chilled 0.1 M LiCl. Radioactivity was determined by scintillation oscillography with 3 ml scintillation solution per filter. Uptake rates are expressed in nanomoles min^{-1} OD_{600}^{-1}. For determination of the kinetic parameters, glucose uptake experiments were conducted with various glucose concentrations (12.5–400 μM). To determine V_{max} and K_m, the data were fit to the Michaelis-Menten equation by a nonlinear regression program and graphed on a Lineweaver-Burk plot. The substrate specificity was studied by glucose uptake competition assay using a 100-fold excess of nonradioactive sugar (10 mM) as described previously [van Wezel et al., 2005]. Transport activity without additional sugar was set to 100% (control).

Computational Analyses

The genome data of *M. smegmatis* mc^2 155 were collected from the primary annotation database (http://cmr.tigr.org/tigr-scripts/CMR/shared/Genomes.cgi) available at The Institute for Genomic Research (TIGR). Protein databank searches were carried out at the BLAST server of the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, Md., USA (http://www.ncbi.nlm.nih.gov), the BLAST server of the Transport Classification Databank, TCDB (www.tcdb.org), and at TIGR. Multiple alignment analysis and graphical optimization were achieved with CLUSTALW (www.ebi.ac.uk/clustalw) and BOXSHADE (www.ch.embnet.org/software/BOX_form.html) software. Hydrophobicity profiles and topology predictions were calculated with the TopPred II software [Claros and von Heijne, 1994]. The phylogenetic tree was calculated with the neighbor joining method as implemented in CLUSTALW. The tree was graphically visualized by importing phylip (*.ph) files into the TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) software that was kindly provided by Rod Page.

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